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A Phenotypic Analysis of *Tetrahymena* Centrin 2

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Abstract

Basal bodies are microtubule organizing centers responsible for anchoring and organizing the cilium. Cilia are found on nearly every mammalian cell type and are important for various cellular functions including fluid movement and sensing the surrounding environment. A widely conserved component known to be important at basal bodies is centrin, a small Ca^{2+} binding protein. Centrins divide into two groups based on sequence comparison to the human centrins, hsCETN2 and hsCETN3. Here, we utilized the ciliate *Tetrahymena* to examine the distinct nature of the two centrin groups through an analysis of Cen2, a hsCETN3 homolog. The results indicate that Cen2 is a basal body component and important for basal body stability and orientation. Our data suggest that the two centrin groups have distinct roles at basal bodies.

Introduction

Basal bodies are microtubule-organizing centers (MTOCs) found at the surface of many cells through-out the animal kingdom and are responsible for organizing and nucleating the cilium (Bornens, 2002). The ubiquitous conservation of cilia along with their wide range of cellular functions, ranging from fluid movement to photo-, chemo-, and mechanosensation indicates that they are important for the cell (Marshall and Nonaka, 2006). Based on functionality, cilia can be divided into two distinct groups: 1) primary cilia and 2) motile cilia. Primary cilia can be found on the large majority of human cells and play an important role in receiving cues from the surrounding environment. The second class of cilia can be found on cells responsible for either creating locomotion or fluid movement like the epithelial cells along the respiratory pathway (Marshall and Nonaka, 2006).

Due to the diverse cellular responsibilities and prominence of cilia in human tissues, mutations resulting in defective basal body or cilia function can lead to many diseases collectively known as ciliopathies (Bandano et al., 2006). These diseases are characterized by a wide-range of pathologies including cystic kidneys, polydactyly, and retinal degeneration. Bardet-Biedl syndrome, a pleiotropic genetic disorder characterized primarily by retinal pigmentosa, obesity, and mental retardation, is caused in part by defects in anchoring the cilia (Bandano, et al., 2006; Kim et al., 2004). The diversity of problems arising from basal body and/or cilia dysfunction emphasizes the need to better our understanding of basal body and cilia function.

Basal bodies are cylindrical microtubule structures composed of nine sets of triplet microtubules, giving the structure nine-fold symmetry (Allen, 1969; Dawe et al., 2007; Pearson and Winey, 2009; Wolfe, 1970). Electron microscope and basal body component localization

studies have revealed that there are distinct regions within the basal body (Figure 1) (Kilburn et

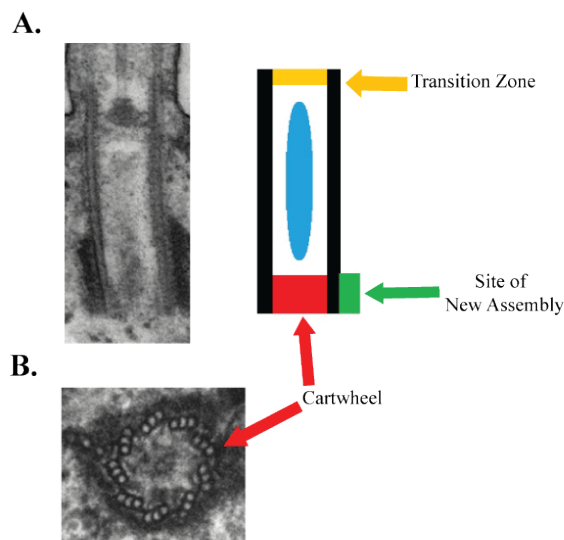


Figure 1: Basal Body Structure. A) Electron micrograph of a longitudinal basal body section. Accompanying schematic illustrates the transition zone, site of new assembly and cartwheel. B) Electron micrograph of a basal body in transverse section to further illustrate the cartwheel and the nine triple microtubules comprising the structure.

al., 2007). The proximal end of a basal body contains the cartwheel and the site of new assembly. The cartwheel structure is an array of nine proteinaceous spokes that anchor and establish the nine-fold symmetry of the basal body (Gavin, 1984; Strnad and Gönczy, 2008). Adjacent to the cartwheel but outside the microtubule scaffold is the site of new assembly where the nascent basal body begins to form. At the distal end of the basal body is the transition zone where the sets of triple microtubules

become the doublets that compose the axenome (Figure 1).

Despite our understanding of basal body morphology, the molecular mechanisms responsible for assembling and maintaining the structure are not well understood. Proteomic studies of the basal body have revealed that it is composed of over 1,000 different proteins (Gherman et al, 2006; Kilburn et al., 2007). Comparative proteomic studies indicate that many of these proteins are evolutionarily conserved and may play similar roles across species (Gherman et al, 2006). One of these conserved components shown to be important at basal bodies is centrin (Salisbury et al., 2002; Stemm-Wolf et al., 2005). Centrins belong to the super family of EF-hand Ca^{2+} binding proteins. They are widely conserved and are expressed in various organisms including humans, yeast, and ciliates (Salisbury et al., 2002; Stemm-Wolf et al., 2005; Weich et al., 1996). Sequence comparisons indicate that there are two distinct groups of centrins based on

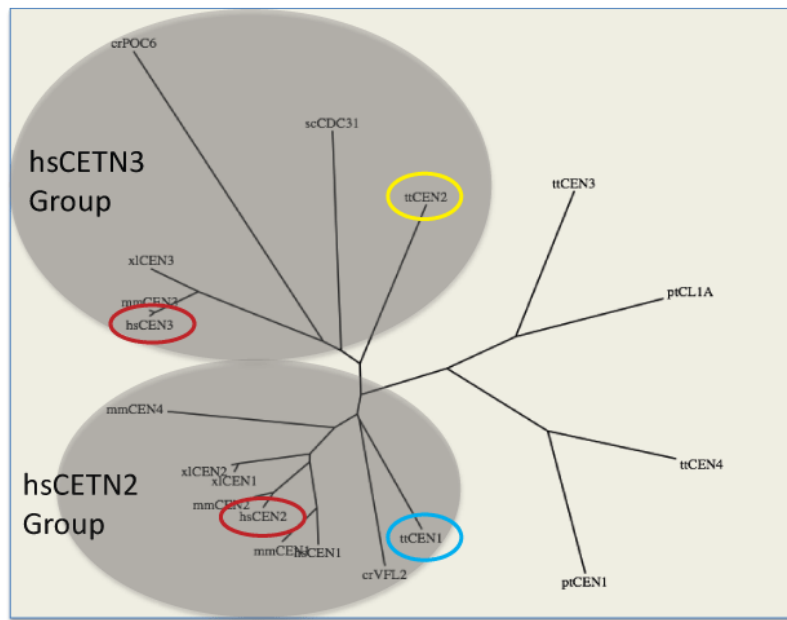


Figure 2: Phylogenetic tree of centrin family members. Based on sequence homology there are two distinct groups of centrins. Tetrahymena has two centrins that localize to basal bodies. ttCen1 (blue circle) belongs to the hsCETN2 group and ttCen2 (yellow circle) belongs to the hsCETN3 group and is the focus of this study. Human homologs of each group are circled in red.

similarity to either human Centrin 2 (hsCENT2) or human Centrin 3 (hsCENT3) (Figure 2). Of the two families, members of the hsCENT2 group have been studied more extensively than their hsCENT3 counterparts. Multiple studies have demonstrated that the centrin

hsCETN2 group is important for the duplication, orientation, and maintenance of basal bodies (Koblenz et al., (2003); Ruiz et al., (2005); Salisbury et al.,

(2002); Stemm-Wolf et al., 2005). Due to the divergent nature of the two centrin groups and a lack of research on the hsCENT3 group, a characterization of hsCENT3 homologs is needed.

We have utilized the unicellular ciliate *Tetrahymena* in order to determine the nature of the two distinct centrin groups. *Tetrahymena* cells are 50µm long and contain about 700 basal bodies per cell (Frankel, 1999). These basal bodies exist in two discrete populations: the cortical rows which run along the length of the cell and the highly organized oral apparatus. Basal bodies along the cortical rows serve to anchor cilia for locomotion while the oral apparatus is a feeding structure (Frankel, 1999). The high number of basal bodies makes it ideal for conducting studies to understand the assembly and morphology of these structures. In addition to the abundance of

basal bodies, *Tetrahymena* are also genetically malleable in a manner similar to the budding yeast, *Saccharomyces cerevisiae*. Through homologous recombination we are able to create complete gene knockouts in *Tetrahymena*, allowing us to study both essential and non-essential genes (Hai et al., 1999).

Tetrahymena has four known centrins and two of these, Cen1 and Cen2, are basal body components (Stemm-Wolf et al, 2005). These centrins belong to the hsCETN2 and hsCETN3 groups, respectively. Cen1 has been studied in *Tetrahymena* and has provided a basic understanding of its role at basal bodies. Ultra-structural examination of Cen1 shows that it localizes to the site of new assembly, directly adjacent to the basal body, and to the distal end of the basal body at the transition zone (Stemm-Wolf et al., 2005). An analysis of the cen1 null (*cen1Δ*) indicates that Cen1 is important for basal body duplication and maintenance; however, the *cen1Δ* is not viable and cannot complete more than 3 cell divisions (Stemm-Wolf et al., 2005). Because Cen1 has provided information about the hsCETN2 group an analysis of Cen2 is needed to elucidate the distinct nature of the hsCETN3 group.

This study aims to elucidate the function of the centrins within the hsCENT3 group through an analysis of *Tetrahymena* Cen2. The data suggests that Cen2 appears to be distinct from Cen1; even though, Cen2 functions in a manner similar to Cen1.

Materials and Methods

Strains and Culture Conditions

The wild-type *T. thermophila* strains B2086 and CU428 (Tetrahymena Stock Center, Cornell University, Ithica, NY) were used in this study as the wild-type comparison strain and as a starting point for the *cen2Δ* strain. Cells were grown in 2% super peptose (2% SPP) media (2% proteose peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% Fe-EDTA) at 30°C or 38°C. To do starvation experiments, cells were grown to mid-log phase in 2% SPP media and washed twice before being resuspended in 10mM Tris-HCl, pH 7.4, at 30° or 38°C for 14-24 hours.

The green fluorescent protein (GFP)-Cen2 strain was generated by cloning *CEN2* into the pENTR-D Gateway Entry Vector (Invitrogen, Carlsbad, CA), followed by cloning the coding sequence into the vector pBSMTTGFPgtw (Dr. Chalker, Washington University, St. Louis, MO) using the Gateway cloning protocol (Invitrogen, Carlsbad, CA). The construct was then biolistically introduced into the macronucleus of the B2086 strain and integrated into the genome by homologous recombination (Cassidy-Hanlet et al., 1997). This construct confers resistance to cycloheximide, adds GFP to the N-terminus of Cen2 and is under the control of the metallothionein inducible promoter.

Experiments with GFP-Cen2 were conducted by inducing GFP-Cen2 expression by adding 0.3 µg/mL of cadmium chloride (CdCl₂) to media. Induction was carried out for 3 hours. After induction, cells were washed once with 10mM Tris-HCl, pH 7.4, concentrated by pelleting and imaged.

***cen2Δ* Generation**

The *cen2Δ* was generated in the manner described by Gorovsky et al. to create a drug-selectable strain that has no functional *CEN2* (1999). To summarize, a construct containing the

NEO2 cassette, which provides paromomycin resistance, flanked by 1.5kb of the regions upstream and downstream of *CEN2* was biolistically transformed into the micronucleus of mating wild-type Tetrahymena strains, B2068 or CU428, and integrated at the *CEN2* locus by homologous recombination (Stemm-Wolf et al., 2005). The resulting heterokaryons were then mated and their micronuclei combined to form new macronuclei with the *NEO2* cassette replacing *CEN2* at the endogenous locus. The *cen2Δ* was selected for by resistance to paromomycin. Total genomic DNA was isolated by phenol:chloroform:isoamyl alcohol extraction and isopropyl alcohol precipitation (Gaertig et al., 1994). PCR confirmed the integration of the *NEO2* cassette and lack of *CEN2*.

Recombinant Cen2

Tetrahymena stop codons differ from canonical stop codons so *CEN2* was optimized for expression in *E. coli* and resynthesized by GenScript (Piscataway, NJ). The optimized *CEN2* sequence was cloned into the *E. coli* expression vector pQE10 that adds a N-terminal 6His-tag to the protein. The resulting vector, pQE10-Cen2 was transformed into the *E. coli* strain M15.

The *E. coli* strain M15 containing pQE10-Cen2 was grown overnight at 38° in Luria Broth + 0.2% glucose with 50 µg/mL kanamycin and 100µg/mL ampicillin. This culture was used to inoculate 500mL of Luria Broth, and cells were incubated at 38° for four hours then shifted to room temperature for an hour. pQE10-Cen2 expression was induced with 300µM IPTG for 3 hours. Cells were pelleted, washed once with phosphate buffered saline (PBS) and stored frozen.

Recombinant Cen2 was isolated on a nickel affinity column as described by Stemm-Wolf et al. (2005). Briefly, pellets were resuspended in PBS with protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and pepstatin) and lysozyme. Cells were sonicated and pelleted. The supernatant was loaded onto a Talon resin column (BD Biosciences

Clontech, Palo Alto, CA) and elutions using PBS + 200mM imiozole were collected. Fractions with Cen2 were pooled and stored frozen in 10% glycerol.

Antibody Generation

The rabbit anti-Cen2 peptide antibody was developed by YenZym Antibodies (San Francisco, CA). The antibody epitope consisted of a peptide containing the first 17 amino acids of the N-terminal tail of Cen2 (MNYSPKANKMKRKLKQEC). The antibody was affinity purified using recombinant Cen2.

Recombinant Cen2 was chemically ligated to AminoLink gel (Pierce Biotechnologies, Rockford, IL) to create an affinity column. The column was prepared by alternating two washes of 0.2M glycine, pH 2.8, and 0.1M NaHCO₃, pH 8.5, 0.5 M NaCl. The crude antibody was prepared by adding 10x TTBS and 4M NaCl and centrifuging the preparation. The crude preparation was incubated for 48 hours with the affinity column at 4°C. Elutions using 0.2M Glycine, pH2.8, 0.02% NaN₃, were collected. Fractions were pooled and dialyzed against PBS-azide at 4°C for 16 hours.

Fluorescence Microscopy

All cell imaging, both live-cell and immunofluorescence, was conducted at room temperature using an Eclipse Ti inverted microscope (Nikon, Japan) fitted with a CFI Plan Apo VC 60x H numerical aperture 1.4 objective (Nikon, Japan) and a CoolSNAP HQ2 charge-coupled device camera (Photometrics, Tuscon, AZ). Metamorph Imaging software (Molecular Devices, Sunnyvale, CA) was used to collect images. Live-imaging exposure for GFP-Cen2 was 200ms. The Metamorph Imaging software was used to analyze images by subjecting them to the nearest neighbors deconvolution algorithm.

Live-cell imaging was conducted to examine cells expressing GFP-Cen2. Cells were washed once with 10mM Tris-HCl, pH 7.4, pelleted, and placed on microscope slides.

All cells to be examined with immunofluorescence were chemically fixed as described by Stuart and Cole with paraformaldehyde and ethanol (2000). Cells were placed onto antibody slides coated with poly-L-lysine (Bellco Glass, Vineland, NJ). Five primary antibodies were diluted in PBS + 1% bovine serum albumin (BSA) and used in this study. The affinity purified rabbit polyclonal *Tetrahymena* Centrin 2 antibody (this study) was diluted 1:100, the rabbit polyclonal *Tetrahymena* Centrin1 antibody (Stemm-Wolf, 2005) was diluted 1:1000, the mouse monoclonal 20H5 antibody raised against the *Chlamydomonas* centrin (Dr. J. Salisbury, Mayo Clinic, Rochester, MN) was diluted 1:1000, and the mouse monoclonal kinetodesmal (KD) fiber antibody, F1-5D8, (Dr. Frankel) was diluted 1:250. Primary incubations were carried out overnight at 4°C, except for the K-antigen antibody, which was incubated at 4°C over three nights. Following the primary incubation, cells were washed 5 times with PBS + 0.1% BSA. Cells were then incubated at room temperature for an hour with secondary antibodies diluted 1:1000 in PBS + 1% BSA. The secondary antibodies used were anti-rabbit FITC, anti-rabbit Texas Red, anti-mouse FITC, and anti-mouse Texas Red (Jackson ImmunoResearch Labs, West Grove, PA). After secondary antibody incubation, cells were washed five times with PBS + 0.1% BSA and mounted with Citifluor (Citifluor, London, United Kingdom).

***cen2Δ* Analysis**

Basal Body Count per Cell: Cells progressing through the cell cycle and cells arrested in starvation media (10mM Tris-HCl, pH 7.4) were analyzed. Cells were stained with the anti-Cen1 antibody to label basal bodies. For cells progressing through the cell cycle, cells staged in G1 were quantified. The “Count Particles” function of the program ImageJ (National Institutes of

Health, Bethesda, MD) was used to count the basal bodies across the whole cell surface area. A total of 25 cells were analyzed for each condition.

Results

Cen2 is a basal body component

To understand the role of Cen2, we first asked if it localized to basal bodies like Cen1. The N-terminal end of the Cen2 gene was tagged with green fluorescent protein (GFP) under the control of a metallothionein promoter. After inducing expression of the construct, GFP-Cen2 localization was observed at basal bodies along the cortical rows and in the oral apparatus (Figure 3A), indicating that Cen2 is a basal body component.

The localization of GFP-Cen2 was examined further by analyzing its incorporation at basal bodies using an assay developed in our lab (Pearson et al., 2009). This assay takes advantage of the inducible promoter to control the expression of the GFP-Cen2 fusion protein. To perform this assay cells were grown to confluence to mimic starvation conditions. In starvation conditions the cells are arrested at G1 and basal bodies are not being duplicated. After 24 hours at confluence, cells were released into media containing cadmium chloride to induce expression of GFP-Cen2 and initiate the assembly of new basal bodies. At 2 hours, before the cells have completed a full cell cycle (Pearson et al., 2009; Pearson and Winey, 2009) we saw two types of incorporation signals. First, like Cen1, we saw bright and dim signals in the middle regions of the cell (Figure 3A). In this region basal bodies are being duplicated with the daughter basal body being assembled anterior to its mother basal body (Frankel, 2000; Kaczanowski, 1978; Perlman, 1973). The bright signal corresponds to the daughter basal body and the dim signal corresponds with the mother basal body. The bright signal indicates that GFP-Cen2 is incorporated into newly assembled basal bodies upon induction of the construct. The dim signals at mature basal bodies indicate that there is also a dynamic population of Cen2 at basal bodies as there is some exchange with the cytoplasmic pool of GFP-

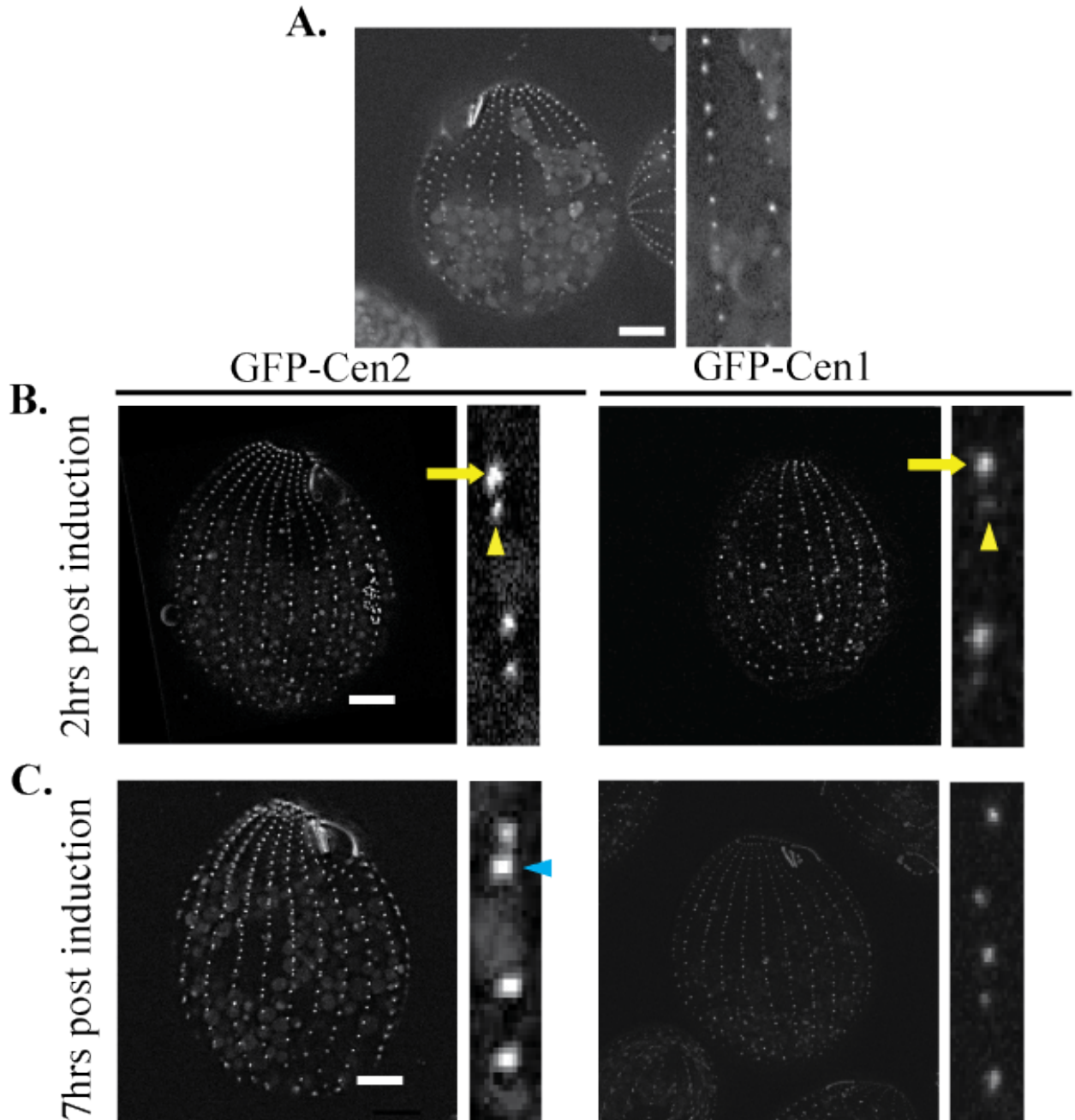


Figure 3: Localization of GFP-Cen2 and GFP-Cen1. A) GFP-Cen2 localization supports that Cen2 is a basal body component. B) At 2 hours after induction GFP-Cen2 exhibits a similar pattern of localization as GFP-Cen1. Both present bright and dim pairs corresponding to mother (arrow head)/daughter (arrow) basal bodies. C) 7 hours after induction GFP-Cen2 localization differs from GFP-Cen1 localization. GFP-Cen2 exhibits non-uniform labelling of mature basal bodies (blue arrow) where as GFP-Cen1 labels all basal bodies evenly. Bars are 10 μ m long.

Cen2 upon expression. This pattern is consistent with the incorporation of GFP-Cen1 (Figure 3B) (Stemm-Wolf et al., 2005).

At 7 hours post-induction cells were visualized again. At this time point, cells have assembled multiple basal bodies in the presence of GFP-Cen2, so we see mainly uniform labeling, except for bright signals at what appear to be mature basal bodies (Figure 3C). This differs from GFP-Cen1 because, at this time point, basal bodies have equal labeling by GFP-Cen1 indicating that all basal bodies, new and old, have equal amounts of Cen1 (Figure 3C) (Stemm-Wolf et al., 2005). This suggests that Cen2 is more concentrated at mature basal bodies than at newly assembled basal bodies, a distinguishing characteristic of Cen2 and not Cen1.

To confirm the unequal labeling of GFP-Cen2 into basal bodies and analyze the localization of endogenous Cen2, we generated an antibody specific to endogenous Cen2 (Figure 4A). The anti-Cen2 antibody confirmed a similar localization pattern for endogenous Cen2 to that seen with GFP-Cen2 (compare figures 3A and 4A).

To better understand the biology of the bright anti-Cen2 signals observed, we examined cycling cells with the k-antigen antibody, which labels only mature basal bodies (Williams et al., 1990; Shang et al., 2005). Co-localization of the anti-Cen2 antibody with the K-antigen indicates that Cen2 is localized at a higher concentration at mature basal bodies than at new basal bodies (Figure 4B). The co-localization of Cen1 with the K-antigen antibody supports that Cen1 is uniformly concentrated at mature basal bodies (Figure 4B). The localization and incorporation studies suggest that Cen2 behaves distinctly from Cen1 at basal bodies.

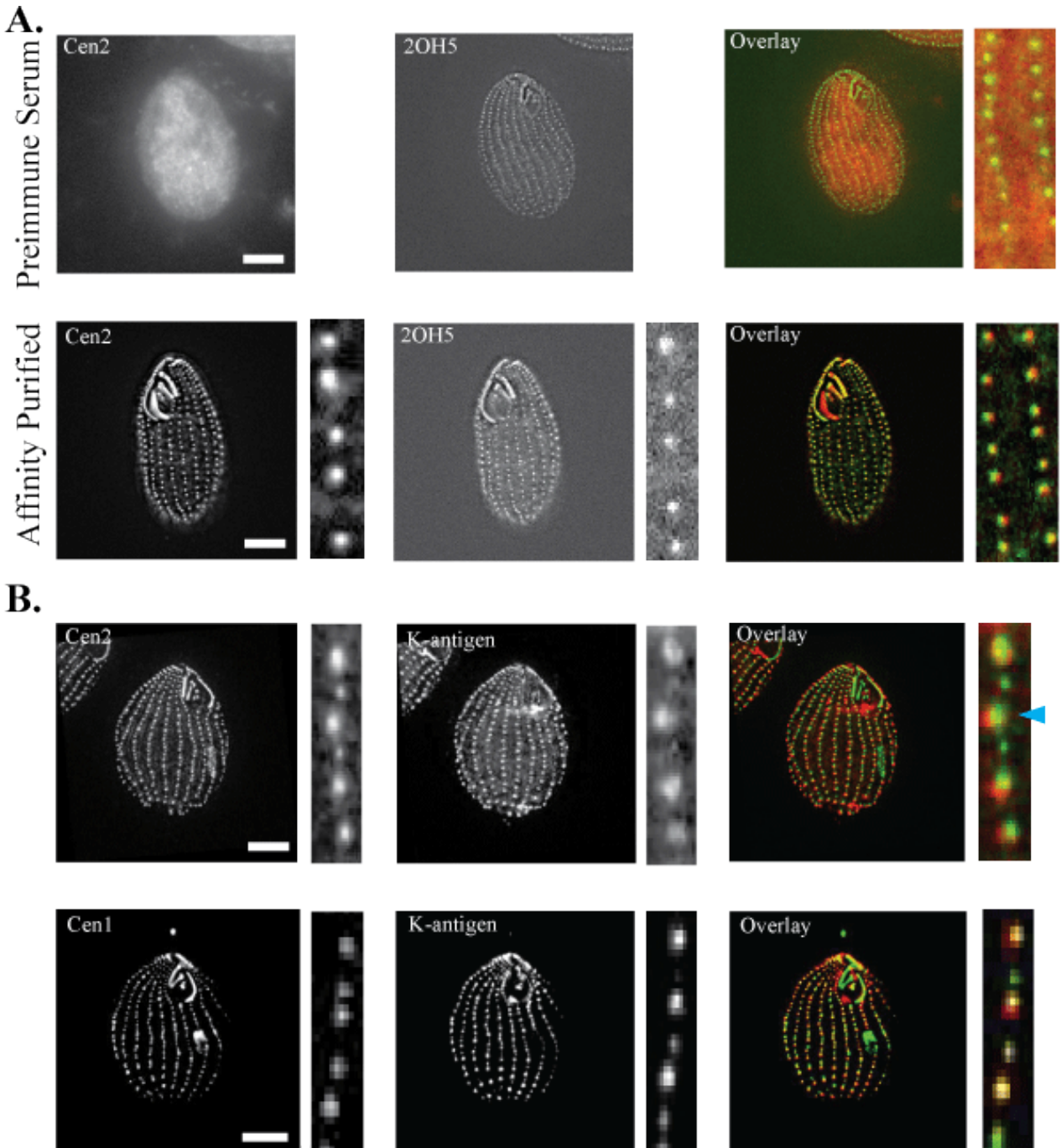


Figure 4: Cen2 Antibody. A) The Cen2 antibody was generated against the N-terminal tail of Cen2. The affinity purified antibody is specific for endogenous Cen2. Co-localization with 20H5 confirms Cen2 is a basal body component. B) Co-localization with the K-antigen antibody indicates that Cen2 is located at a higher concentration at mature basal bodies (blue arrow). Cen1 co-localization suggests that Cen1 is equally concentrated at all basal bodies. Bars are 10 μ m long.

The *cen2Δ* has basal body orientation defects and loss of basal bodies

To determine whether or not the two groups of the centrin family have distinct functions we created a strain lacking the *Cen2* gene. Through homologous recombination (see Materials and Methods) we replaced the coding sequence of *CEN2* with the *NEO2* cassette. The *cen2Δ* was confirmed by using PCR to verify that the *NEO2* cassette was present in the genome and that *CEN2* was absent (Figure 5A). To further confirm that *Cen2* was absent in the strain we examined cells by immunofluorescence using the centrin antibody, 20H5, and the affinity purified *Cen2* antibody. In wild-type cells the *Cen2* antibody co-localized with 20H5; however, in the *cen2Δ* the *Cen2* antibody had no specific labeling (Figure 5B).

Cells were grown to mid-log phase and examined for basal body defects. We observed that a significant population of cells (89%) have gaps and branching of the cortical rows (Figure 6A). At the posterior ends of the gaps the kinetodesmal (KD) fiber, a nonmicrotubule based structure that points towards the end of the cell, is improperly oriented (Figure 6A) (Allen, 1969). In wild-type cells the KD fibers extend towards the anterior of the cell, maintaining properly aligned basal bodies along the cortical rows (Figure 6A). We also observed another defect related to the KD fibers in the null. In wild-type cells each basal body has a single KD fiber extending from it, but in the *cen2Δ* we observed basal bodies with two KD fibers extending from it. The two KD fibers result in a branching of the cortical row and a new, improperly aligned cortical row (Figure 6A). This result indicates that *Cen2* may play a role in basal body orientation and/or in the assembly process. Because we observed gaps in the cortical rows we asked if the *cen2Δ* had fewer basal bodies.

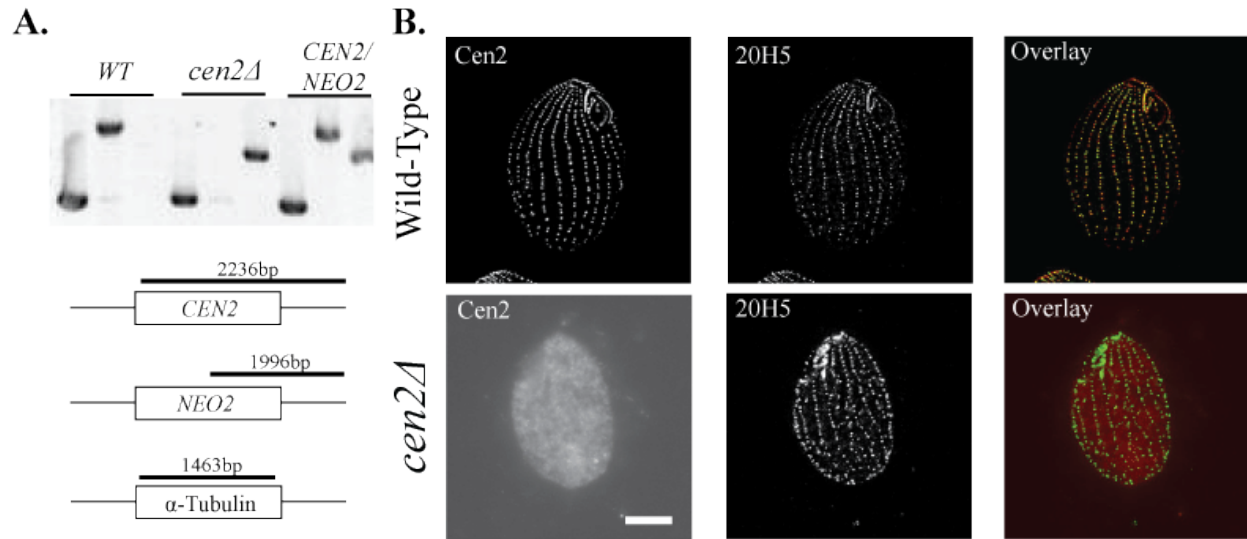


Figure 5: *cen2Δ*. A) PCR verification of *cen2Δ* compared to wild-type the *CEN2/NEO2* heterokaryon. *CEN2* was replaced by the *NEO2* cassette and PCR confirms the absence of *CEN2*. The heterokaryon has both wild-type *CEN2* and the *NEO2* cassette. Lane 1 of each set is α -tubulin. Lane 2 is wild-type *CEN2*. Lane 3 is *NEO2*. B) Immunofluorescence confirmation of the *cen2Δ*. Wild-type cells have specific labeling with the Cen2-antibody. The *cen2Δ* has no specific labeling of Cen2 further confirming the absence of *CEN2* in the genome. Bars are 10 μ m long.

Cells were grown to mid-log phase at permissive temperature, 30°C, and examined by immunofluorescence using the Cen1 antibody which labels all basal bodies. The *cen2Δ* was compared to wild-type cells by analyzing the number of basal bodies per cell. This comparison revealed that the strain lacking Cen2 had significantly fewer basal bodies (461 ± 54 v. 630 ± 86 ; $p < .001$).

In all, our analysis of the *cen2Δ* revealed that there are basal bodies orientation defects and a loss of cortical row basal bodies, suggesting that Cen2 is important in these processes.

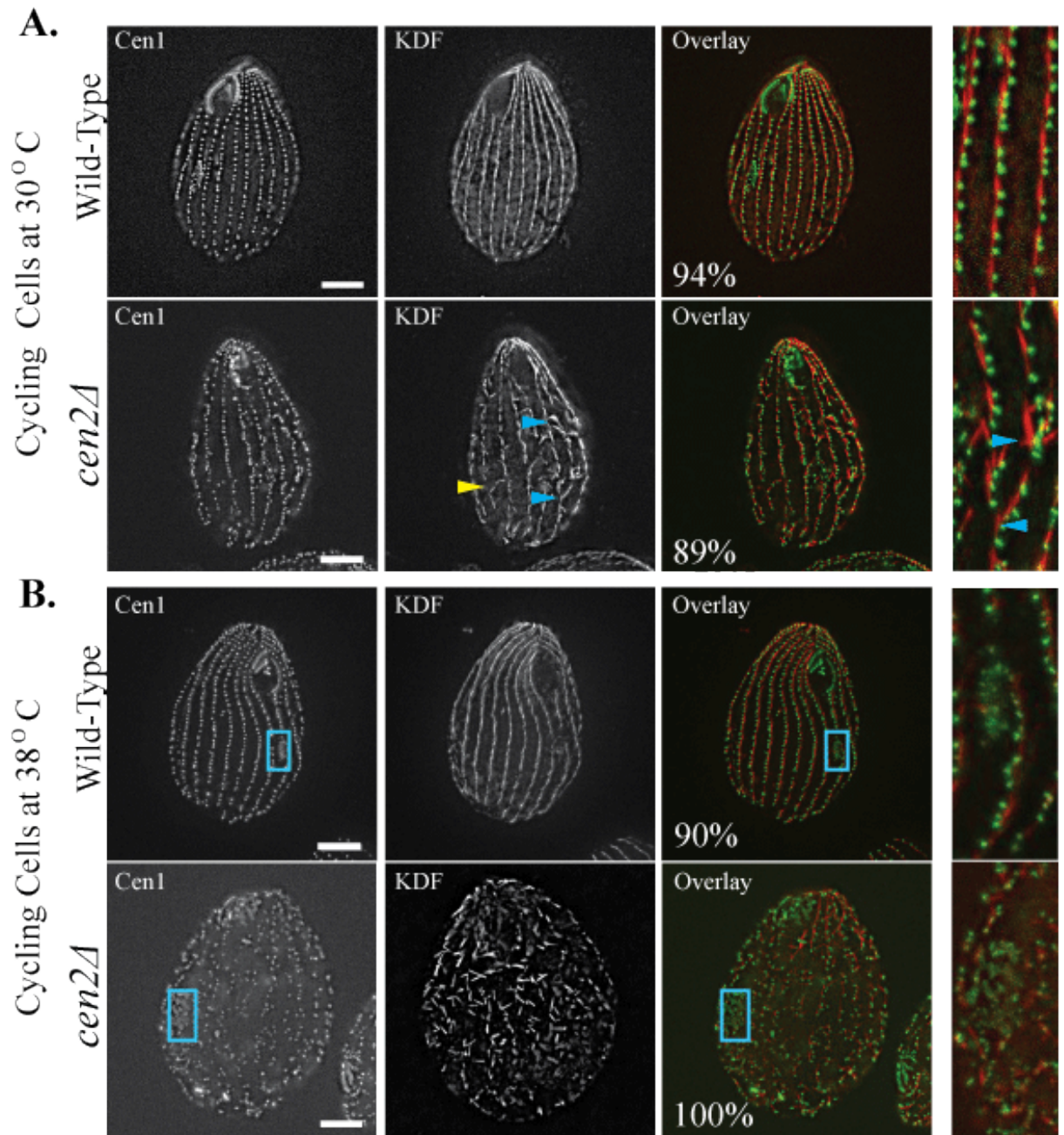


Figure 6: Cycling cells have phenotypes at permissive and non-permissive temperatures. A) Cells at permissive temperature have gaps within the cortical rows due to improperly aligned KD fibers (yellow arrow) and have branching of cortical rows (blue arrows). B) Cells at non-permissive temperature display an increase in cortical row disorganization and the oral apparatus is deteriorating (white arrow) but the presence of an oral primordium (blue boxes) indicates that they are able to assemble new basal bodies. Insets are 9um wide. Percentages indicate frequency of observed phenotype for 100 cells. Bar inserts are 10um.

The *cen2Δ* has increased basal body orientation defects and loss of basal bodies at non-permissive temperature

To examine if the lack of Cen2 resulted in temperature sensitive phenotypes cells were grown in SPP media for 24 hours at 38°C, fixed, and stained for immunofluorescence microscopy. These cells are larger than those at permissive temperature, as is wild-type but the *cen2Δ* is unable to maintain cortical row organization (Figure 6B). At permissive temperature, the KD fibers of the *cen2Δ* occasionally branch from the cortical rows but the overall organization of basal bodies into linear rows is somewhat maintained (Figure 6A). However, at non-permissive temperature there is no evidence of cortical rows and the KD fibers are pointing in all directions in the null (Figure 6B). This observation further suggests that Cen2 is important for basal body orientation.

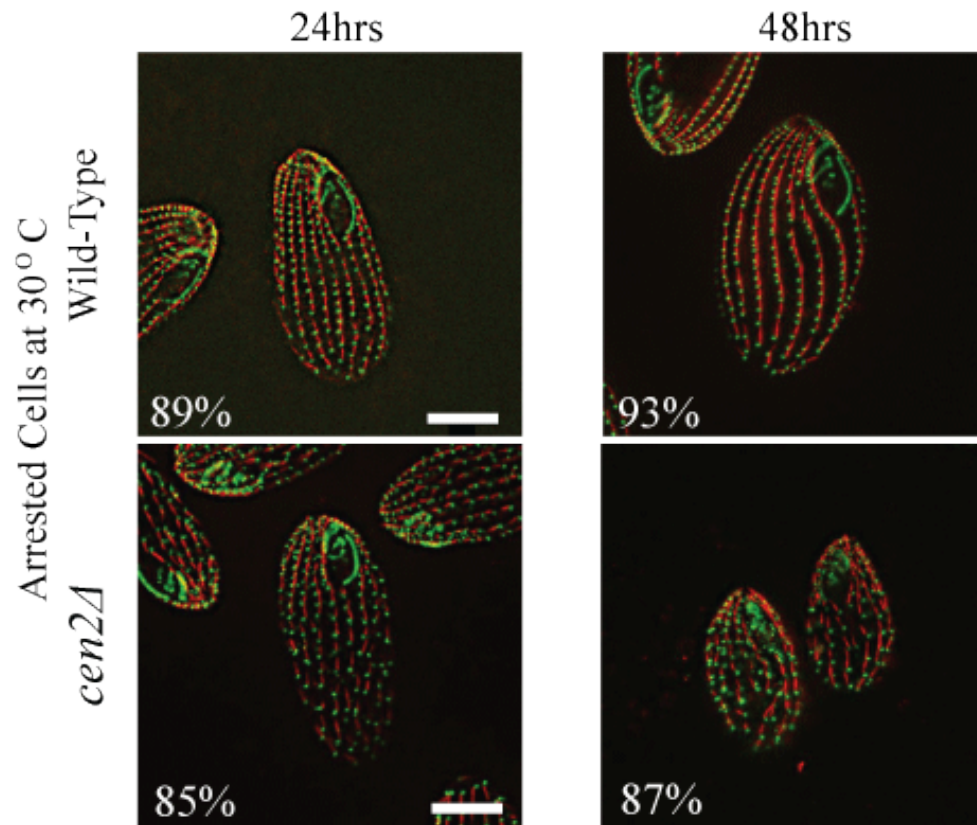
In addition to a loss of organization, these cells also appear to have fewer basal bodies than wild-type cells. Due to the phenotypes observed, we hypothesize that a maintenance defect is responsible for this phenotype as we observe a developing oral primordium, which is indicative of new basal body assembly (blue box, Figure 6B).

The *cen2Δ* has basal body maintenance defects

To determine if the *cen2Δ* has a basal body maintenance defect, we examined cells in starvation conditions. By shifting cells to starvation media we are able to inhibit new basal body formation while the cells only maintain their current basal bodies. Both wild-type and *cen2Δ* cells were incubated in starvation media at 30°C for 24 and 48 hours. After 24 hours, *cen2Δ* cells do not exhibit any defects from cycling cells at 30°C (Figure 7A). However, after 48 hours cells

A.

Time In Starvation Media



B.

Basal Bodies per Cell				
Time in Starvation	24 hours	48 hours	Rate of Basal Body Loss per Hour	
Wild-Type	509+122	504+114	0.24	p=0.8613
<i>cen2Δ</i>	435 ± 98	300± 88	5.64	p=0.0001

Figure 7: The *cen2Δ* has a maintenance defect. A) Under starvation conditions the *cen2Δ* is able to maintain oral apparatus and cortical row organization at 24 hours. After 48 hours the cells have a loss of cortical row organization and the oral apparatus is falling apart. KD fibers are red and Cen1 is green. B) The *cen2Δ* losses basal bodies at a significantly greater rate than wild-type cells. Percentages indicate frequency of observed phenotype for 100 cells. Bars are 10μm long.

exhibited a significant decrease in basal body number, a characteristic not exhibited by wild-type cells (Figure 7A). We determined that the rate of basal body loss per hour in the null was much greater than wild-type (0.243 vs. 5.641) (Figure 7B). This result indicates that the *cen2Δ* has problems maintaining mature basal bodies in comparison to wild-type cells. *cen2Δ* cells do not only have fewer basal bodies but there is an increase in cortical row disorganization, another feature not exhibited by wild-type cells which maintain correct positioning of the KD fibers over 48 hours (Figure 7A).

Our results from the starvation study suggest that Cen2 is responsible for maintaining basal bodies. The phenotypes observed with the KD fibers indicate improperly oriented and rotated basal bodies suggesting a role for Cen2 in orienting basal bodies along the cortical rows.

Discussion

In this study we performed an analysis of the *Tetrahymena* Cen2 in an effort to better understand the function of the hsCETN3 group. We observed that Cen2 has an exchangeable population at basal bodies and has a higher concentration at mature basal bodies compared to immature basal bodies. We also analyzed the *cen2* null strain and observed there are two distinct phenotypes associated with the lack of Cen2: basal body stability and orientation defects. This suggests that the role of Cen2 at basal bodies is to properly align basal bodies and to stabilize basal bodies.

Another goal of this study was to inform our knowledge on how the two groups of centrins are different from each other. Our results from the localization studies and incorporation studies suggest that the two *Tetrahymena* centrins, Cen1 and Cen2, are recruited to basal bodies in a different manner. Cen2 and Cen1 both incorporate at new basal bodies and have a dynamic exchange at basal bodies. However, there is a localization difference for Cen1 localizes to mature and newly assembled basal bodies evenly but Cen2 localizes to mature basal bodies at a higher concentration, suggesting that Cen2 may have a role in basal body maturation. Together, the localization differences observed between Cen1 and Cen2 suggest that they share similar roles in orienting the basal body while they may have different roles at mature basal bodies.

The results of the phenotypic analysis of the *cen2Δ* suggest that the two groups may behave differently from each other. Studies investigating the *Tetrahymena* hsCETN2 homolog, Cen1 mutants, found that Cen1, like Cen2, is crucial for proper basal body orientation and maintenance. The analysis of the Cen1 mutants also revealed that Cen1 is important for proper separation of newly assembled basal bodies (Vonderfecht, 2010). Though the phenotypes observed do not implicate Cen2 as directly affecting duplication it does appear that Cen2, like

Cen1, is important for proper alignment of the basal bodies within the cortical rows. Proper alignment of basal bodies is crucial, for example, in vertebrate epithelial cells which maintain directional ciliary beating such as those along the oviduct and trachea (Boisvieux-Ulrich et al., 1991; Marshall and Kintner, 2008; Salathe and Bookman, 1995). Without proper alignment the cilia cannot coordinate efficient movement which in the cases described above can result in failure to transport the unfertilized egg or control fluid movement, respectively. We also observed that the absence of Cen2 results in maintenance defects, a phenotype shared with Cen1. The ability to stabilize basal bodies over time is likely important for multiciliated vertebrate cells to function properly. In the ciliopathies, like Bardet-Biedl syndrome where there is a loss of basal bodies and cilia, there is a progressive deterioration of organ systems reliant on cilia and basal bodies such as the kidneys and retinas (Ansley et al., 2003).

Though we saw similar phenotypes for Cen2 and Cen1, there is an important difference between the knockout strains. The *cen2Δ* is viable and stable over many generations while the *cen1Δ* is lethal due to a complete loss of basal bodies (Stemm-Wolf et al, 2005). This important distinction suggests that the two centrin groups are not redundant even though it appears that they serve similar functions.

Continued study is needed to determine the distinct nature of the hsCETN2 and hsCETN3 groups. Research on the hsCETN2 group has focused on the Ca^{2+} binding of centrins as it appears to play an important role in conformation changes for various EF-hand proteins (Drake et al., 1997). This strategy for Cen2 would help elucidate not only how Ca^{2+} functions in regulating the function of the hsCETN3 homologs but further define the divergent nature of the two groups.

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